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VIROLOGY

Virology 309 (2003) 190–195

www.elsevier.com/locate/yviro

Rapid Communication

Limited evolution of West Nile virus has occurred during its southwesterly spread in the United States

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Received 6 September 2002; returned to author for revision 29 October 2002; accepted 3 January 2003

Abstract

Analysis of partial nucleotide sequences of nine West Nile virus strains isolated in southeast Texas during June–August 2002 revealed a maximum of 0.35% nucleotide variation from a New York 1999 strain. Two sequence subtypes were identified that differed from each other by approximately 0.5%, suggesting multiple introductions of virus to this area. Analysis of sequences from cloned PCR products for one strain revealed up to 0.6% divergence from the consensus sequence at the subpopulation level. The presence of unique patterns of small numbers of mutations in North American West Nile strains studied to date may suggest the absence of a strong selective pressure to drive the emergence of dominant variants.

Introduction

West Nile (WN) virus, a member of the Japanese encephalitis (JE) serocomplex of the family *Flaviviridae*, genus *Flavivirus*, was first isolated in the United States in New York City during the summer of 1999. Comparative nucleotide sequencing of WN virus strains from various regions of Africa, Europe, Asia, and Australia identified two major genetic subtypes designated lineages I and II; the New York City WN virus isolate was most closely related to lineage I strains isolated in 1997 and 1998 in Israel (Lanciotti et al., 1999; Briese et al., 2002). This group of viruses has recently been labeled lineage I subtype 2a (Briese et al., 2002). During the summers of 2000 and 2001, WN virus was detected in an expanding geographical range that encompassed most of the eastern United States and southern Canada. In early June 2002, WN virus was isolated in Texas from dead birds collected in the Houston metropolitan area (Harris County). Prior nucleotide sequencing and analysis of WN virus strains isolated in the northeastern United States (New York, New Jersey, Maryland, and Connecticut) during 1999 and 2000 revealed little variation ($\leq 0.35\%$) from the original 1999 New York City isolates (Anderson et al., 2001; Ebel et al., 2001; Lanciotti et al., 2002). In order to determine the extent of genetic variation and evolution of WN virus as it spreads in North America, we compared partial nucleotide sequences from nine recently isolated Texas strains with previously published sequences of strains isolated in the northeastern United States. Our strains were obtained from three different bird species [blue jay (*Cyanocitta cristata*), American crow (*Corvus brachyrhynchos*), and rough legged hawk (*Buteo lagopus*)] and a mosquito pool collected in the Houston area. In addition, we compared sequences of cloned PCR products from a single strain to determine the extent of variation within the virus population of that strain.

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doi:10.1016/S0042-6822(03)00150-8

Table 1
Locations of variable nucleotides in sequences of the premembrane and envelope (prM-E) genes and NS5/3′-noncoding region junction of Houston area 2002 West Nile virus strains compared with the New York 1999 prototype strain 382–99

Strain	Source	Collection date	Template origin[b]	prM-E (nucleotides 466–2469)[a]															NS5/3′ NCR (10141–10785)	
				621	660	679 (prM72)	969	1065	1137	1192 (E76)	1356	1407	1442 (E159)	1729	2154	2392 (E476)	2400	2466	10596	10774
382–99	Flamingo	1999		A	C	U (Ser)	C	C	C	A (Thr)	C	U	U (Val)	U	U	G (Ala)	U	C	A	C
113	Bluejay	06/11/02	B/V		U								C (Ala)			A (Thr)		U		U
114	Bluejay	06/10/02	B/V		U			U	U				C					U	G	U
119	Bluejay	06/14/02	B/V		U								C					U		
123	Hawk	06/20/02	B		U								C					U		U
135	Crow	06/20/02	B		U							C	C					U	—	—
V1151	Mosquito	06/15/02	M		U								C					U	—	—
227	Bluejay	07/05/02[c]	V		U								C	C				U	—	—
362	Bluejay	07/19/02	V			A (Thr)	U			G (Ala)	U				C		C		—	—
476	Bluejay	08/02/02	V	G		A	U			G	U				C		C		—	—

[a] Nucleotide numbers correspond to those of strain 382–99 (Genbank Accession AF196835); in brackets for individual residues are the deduced amino acid substitutions.

[b] B, brain homogenate; M, mosquito pool; V, Vero cell-derived virus. Tissue homogenate and Vero-derived templates were sequenced for strains 113, 114, and 119 and no differences were identified.

[c] Date of sample processing; collection date was during the previous 7 days. Gaps indicate no change from the strain 382–99 sequence; dashes (−) indicate no sequence data were obtained for this region.

Results

Complete premembrane-envelope (prM-E) gene sequences (nucleotides 466–2469) were obtained for six Harris County strains: 113, 114, 119, 123, 135, and V1151 (Genbank Accession Nos. AY185906–AY185911; see Table 1 for details of all strains analyzed and Fig. 1 for collection localities). Alignment of these sequences with the homologous region of the prototype New York 1999 strain 382–99 (Genbank Accession No. AF196835) revealed a maximum of 0.25% nucleotide divergence (mean, 0.18%) over the 2004 nucleotides examined (i.e., prM and E genes; Table 2).

All six strains shared 3 nucleotides (660, 1442, 2466) that differed from the strain 382–99 sequence. Only 1, U→C at 1142, was a coding substitution (Val→Ala at E159; Table 1). Additional divergent nucleotides were identified in each of strains 113 (1 nucleotide encoding an amino acid substitution at E476), 114 (two noncoding changes), and 135 (one noncoding change). Having identified the presence of a conserved coding substitution within the E gene, this region was sequenced for four additional strains collected in Harris County and the amino acid substitution was conserved in all four (data not shown). Sequences of a fragment covering the NS5/3′ noncoding region junction (residues 10141–10785), a region which shows considerable variability between WN virus subtypes (Beasley et al., 2002), were obtained for strains 113, 114, 119, and 123 (Genbank Accession Nos. AY187012–AY187015). These were also highly conserved, with a maximum of two changes from the 382–99 sequence observed over the 645 nucleotides examined (Table 1).

Subsequently, prM-E sequences were obtained for three additional strains from Montgomery County (strain 227), which is located north of Harris County, and the Bolivar Peninsula (strains 362 and 476), located approximately 50 miles southeast of Houston in Galveston County (Table 1; Fig. 1). Strain 227 (Genbank Accession No. AY185912) was almost identical to the Harris County strains; it shared the three conserved nucleotide changes but had an additional unique noncoding change at residue 1729. In contrast, strains 362 and 476 (Genbank Accession Nos. AY185913 and AY185914) shared none of the changes identified in other Houston area strains and differed from these strains by 0.45–0.60% at the nucleotide level (Table 2). Their mean divergence from strain 382–99 was 0.32% and two of the nucleotides that differed from the 382–99 sequence encoded amino acid substitutions at prM72(Ser→Thr) and E76(Thr→Ala) (Tables 1 and 2).

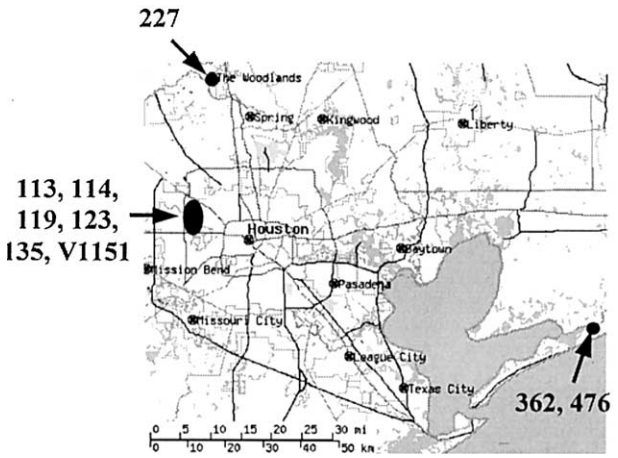

Fig. 1. Map of the Houston area showing collection sites for the West Nile virus strains analyzed in this study. Map drawn using the U.S. Census Bureau's TIGER Mapping Server (http://tiger.census.gov/).

Table 2
Nucleotide sequence divergence (%) of prM and E genes between WN virus strains isolated in and around Houston, Texas, during June–August 2002, selected other North American isolates (382–99 Genbank Accession AF196835; NY00 grouse AF404755; NY00 crow AF404756; NJ00 AF404754; CN99 AF206518), and lineage I strains Israel 1998-stork (ISR98; AF481864) and Egypt101 (E101; AF260968)

Strain	382–99	113	114	119	123	135	V1151	227	362	476	NY00 grouse	NY00 crow	NJ00	CN99	ISR98	E101
382–99	—															
113	0.20	—														
114	0.25	0.15	—													
119	0.15	0.05	0.10	—												
123	0.15	0.05	0.10	0.00	—											
135	0.20	0.10	0.15	0.05	0.05	—										
V1151	0.15	0.05	0.10	0.00	0.00	0.05	—									
227	0.20	0.10	0.15	0.05	0.05	0.10	0.05	—								
362	0.30	0.50	0.55	0.45	0.45	0.50	0.45	0.50	—							
476	0.35	0.55	0.60	0.50	0.50	0.55	0.50	0.55	0.05	—						
NY00 grouse	0.05	0.25	0.30	0.20	0.20	0.25	0.20	0.25	0.35	0.40	—					
NY00 crow	0.15	0.35	0.40	0.30	0.30	0.35	0.30	0.35	0.45	0.50	0.10	—				
NJ00	0.05	0.25	0.30	0.20	0.20	0.25	0.20	0.25	0.35	0.40	0.10	0.20	—			
CN99	0.20	0.40	0.45	0.35	0.35	0.40	0.35	0.40	0.50	0.55	0.25	0.35	0.25	—		
ISR98	0.10	0.30	0.35	0.25	0.25	0.30	0.25	0.30	0.30	0.35	0.15	0.25	0.15	0.30	—	
E101	4.29	4.49	4.54	4.44	4.44	4.39	4.44	4.49	4.29	4.34	4.34	4.44	4.34	4.49	4.19	—

Comparison of these sequences with those of other WN virus isolates made in the northeastern United States during 1999 and 2000 (Anderson et al., 2001; Ebel et al., 2001; Lanciotti et al., 2002) revealed that the mutations identified here are new and unique. Nucleotide sequence divergence between the 2002 Texas isolates and several other North American isolates from 1999/2000, for which complete prM-E gene sequence data were available, ranged between 0.20 and 0.55%, with strains 362 and 476 the most divergent from all other North American isolates sequenced to date (Table 2; data for comparisons with sequences of other North American WN isolates not shown but available from the authors on request). Comparisons of the sequences of these strains with the Israel 1998-stork strain (Genbank Accession No. AF481864), identified as being the closest relative of the virus introduced to North America (Lanciotti et al., 1999), and with E protein gene sequences of other lineage I subtype 2a Israeli isolates obtained between 1997 and 2000 revealed that strains 362 and 476 were almost equally divergent from 382–99 and Israel98 and no more closely related to any other sequences (Table 2 shows divergence data for comparisons with strain Israel98; data for other comparisons not shown).

Consistent with the results of earlier studies (Anderson et al., 2001; Ebel et al., 2001) no differences were found between prM-E gene sequences obtained directly from tissue homogenates of infected bird brain or from Vero cell-passaged (1×) virus templates for strains 113, 114, and 119. Although we have not examined complete genome sequences, a single cell culture passage does not appear to have selected for sequence variants or induced additional mutations in the WN virus genome.

To assess the degree of nucleotide sequence divergence within a single strain, the WN1101/WN1816A PCR product derived from strain 114 tissue homogenate was cloned into pGEM-T Easy (Promega Corp., Madison WI); 20 individual clones were sequenced and a 660-bp region (nucleotides 1036–1795) was compared to the consensus sequence obtained by directly sequencing the PCR product. This fragment included the U→C (E159 Val→Ala) mutation at nucleotide 1442. Eleven clones were identical to the consensus sequence, and all 20 contained the E159 coding substitution at nucleotide 1442 and the strain-114-specific change at nucleotide 1137. Changes were identified at a total of 15 different nucleotides in 9 clones (Table 3). These included a nucleotide deletion in 1 clone and the generation of an in-frame stop codon in another, presumably representing nonviable genomes. Six of the other 13 changes would have resulted in amino acid substitutions. Nucleotide sequence divergence from the consensus for individual clones was up to 0.61% (mean, 0.12%).

Previously we have shown that WN virus strains isolated in New York during 1999 were highly virulent in a mouse neuroinvasion model. These strains killed 3- to 4-week-old NIH Swiss mice, with average survival times of approximately 7 days and LD_{50} values of less than 1 PFU (Beasley et al., 2002). When strains 114 (Harris County subtype) and 476 (Galveston County subtype) were inoculated intraperitoneally into mice, they caused significant mortality, with calculated LD_{50} values of 4.2 and 1.8 PFU, respectively. Average survival times after a 1000-PFU challenge were 7.5 ± 1.2 and 8.6 ± 0.7 days, respectively.

Discussion

The arrival and spread of WN virus in North America, an event that presumably resulted from a single introduction,

Table 3
Nucleotides and their deduced amino acids that varied between individual clone sequences of a fragment of the E protein gene (residues 1136–1795) of West Nile virus strain 114

Clone[a]	Nucleotide[b]														
	1151 (E62)	1224	1252 (E96)	1299	1361 (E132)	1482	1513 (E183)	1539	1551	1584	1601 (E212)	1696 (E244)	1767	1770	1786 (E374)
Consensus	U (Leu)	U	G (Val)	A	A (Lys)	G	U (Tyr)	A	G	U	U (Leu)	G (Glu)	U	A	U (Phe)
1							C (His)								
2					G (Arg)	A			del.		C (Ser)				
3								G						G	
4										C		A (Lys)	C		
7															C (Leu)
13	A (Stop)			G											
14		C													
17			U (Leu)												
20													C		

[a] Clones 5,6, 8–12, 15, 16, 18, and 19 were all identical to the consensus sequence.
[b] Nucleotide numbers correspond to those of the 382–99 sequence (Genbank Accession No. AF196835).

has allowed a unique opportunity to investigate the adaptation and evolution of an RNA virus as it occupies a new geographical niche. This is the first study to examine, at the nucleotide level, WN virus isolates from the southern United States and to characterize isolates made more recently than the summer of 2000. We have examined approximately one fifth of the genome for several strains, and the results described here mirror those of earlier studies which examined isolates made in the northeastern United States during 1999 and 2000 (Anderson et al., 2001; Ebel et al., 2001; Lanciotti et al., 2002) in that only very limited variability was observed, with a maximum nucleotide divergence of approximately 0.3% from the prototype New York 382–99 strain, although divergence between these and other more recent isolates approached 0.6% in some cases (Table 2).

Among the southeast Texas strains described here, we have identified two distinct nucleotide sequence variants with different local distributions—strains from Harris and Montgomery counties and strains from the Bolivar Peninsula in Galveston County—suggesting there were at least two separate introductions of WN virus into the region. As these strains become established and more local transmission occurs, we anticipate that their distributions will begin to overlap. No comparable sequence data from other southern United States isolates has yet been published, making the identification of specific origins for these Houston area strains impossible at this time. Movement from neighboring Louisiana, which first reported the presence of WN virus during 2001, is likely. However, the Bolivar Peninsula is a wetlands area that hosts large numbers of migrating birds, and it is possible that the strain circulating there could have been carried from a more distant origin.

The fact that none of the nucleotide changes or amino acid substitutions described here were reported in earlier analyses of northeastern United States isolates of WN virus supports the observation that genetically distinct populations are emerging as the virus expands its range. Interestingly, the majority (12 of 17) of nucleotide changes described here were U↔C transitions; this bias has also been noted in the studies of Ebel et al. and Anderson et al., where 9 of 10 and 21 of 31 changes, respectively, were U↔C.

Comparisons among sequences of lineage I subtype 2a strains isolated in Israel between 1997 and 2000 have reported similar levels of nucleotide divergence (up to 0.3% between strains), although these results may be complicated by the possible introductions of closely related subtype 2a strains into Israel during 1997 (or earlier) and 1998 (Hindiyeh et al., 2001; Briese et al., 2002; Malkinson et al., 2002). In addition, the evolutionary picture in Israel is further complicated by cocirculation of lineage I subtype 2b strains during these times (Hindiyeh et al., 2001; Briese et al., 2002).

One or two amino acid substitutions in the prM/E region were identified in each of the strains examined here, which is consistent with previous reports. The mutation at E159 in Harris and Montgomery County strains was of some interest as amino acid differences at this residue have been reported in some African and European strains of lineage I (Lanciotti et al., 2002). Residue E159 is located in E protein structural domain I in a region known to contain important neutralizing epitopes in other flaviviruses (Mandl et al., 1989; Ryman et al., 1997a). Although similar conservative substitutions have been associated with antigenic and phenotypic changes in yellow fever (Ryman et al., 1997b) and other viruses (Mateu et al., 1990; Sitbon et al., 1991; Roper and Moss, 1999) whether this mutation, or any of the other amino acid mutations identified here, reflects a selective adaptation or a random drift in the virus sequence remains to be determined. The WN virus type circulating in North America has been associated with significant mortality in avian species and has been reported to be highly neuroinvasive in mouse and hamster models (Kramer and Bernard, 2001; Xiao et al., 2001; Beasley et al., 2002). The majority

of the Texas strains described here were isolated from sick or dead birds, and testing of two strains (114 and 476) in mice indicated that they retain the strong neuroinvasive phenotype of the original New York 1999 isolates.

The presence of single amino acid substitutions, as have been reported in the E protein sequences of WN strains in the United States until this time, is unlikely to have any significant impact on the efficacy of WN vaccines currently in use or in development; other WN virus subtypes that can be distinguished in cross-neutralization tests differ by approximately 10 to 20 amino acids in the E protein (Lanciotti et al., 2002).

The results of this study and of earlier reports suggest a gradual sequence drift leading to the emergence of sequence variants that have accumulated unique patterns of mutations; as with previous studies (Anderson et al., 2001; Ebel et al., 2001; Lanciotti et al., 2002) we have not identified a widely represented mutation/s that might suggest a strong selective or adaptive process. Comparable nucleotide sequence analysis of strains representing the complete temporal and spatial distribution of WN virus within the United States will be necessary to further our understanding of the evolutionary processes that are occurring as the virus spreads. The presence of unique patterns of mutations, as described here, should also allow the temporal and spatial tracking of genetic variants and provide insights into the mechanisms by which WN virus has spread over such a large area so rapidly.

RNA viruses, including the flaviviruses, exist as "quasispecies"—mixtures of closely related but genetically diverse populations that derive from the relatively high error rates which occur during replication of their genomes. The degree of diversity and the potential for evolution within such a population at any given time is a product of the balance between selection (positive or negative pressures which impact the relative fitness of variants) and genetic drift (the accumulation of random mutations during replication) (Domingo et al., 1998). Here we have shown that within a single WN strain there is considerable nucleotide sequence variation, with almost half of the clones examined differing from the consensus sequence by at least one nucleotide (in a 660-bp region) and almost one third encoding an amino acid substitution. Mean nucleotide sequence divergence from the consensus for all 20 clones was 0.12%, which is comparable to data recently reported for strains of dengue type 3 virus sequenced directly from the sera of human patients (Wang et al., 2002) and from tissue culture-passaged populations (Wittke et al., 2002). The identification of nonviable genomes, containing frameshift deletions and/or substitutions encoding stop codons, is not surprising given the lack of fidelity associated with the replication of RNA virus genomes. The sequence clone that contained the nucleotide deletion was the most divergent from the consensus (four mutations) and possibly represents a very low-fidelity replication event. Consistent with our results, Wang et al. (2002) reported that 5.8% of dengue 3 quasispecies sequences in the serum of human patients represented defective genomes.

We have previously reported the selection of monoclonal antibody neutralization escape variants of WN strain 385–99, a 1999 New York City isolate closely related to strain 382–99 (Beasley and Barrett, 2002). Albeit the product of a relatively artificial in vitro process, each variant was selected as a single plaque pick and represented approximately 0.3% of the wild-type population (based on reductions in titer of the wild- type population in the presence of the selecting antibody). The amount of virus inoculated during mosquito feeding on a host is variable, but for flaviviruses is probably in the order of 100 to 10,000 PFU (Davis, 1934; McLean et al., 1975). Therefore, low-frequency variants with potential for emergence in the face of immune and other selective pressures are probably retained within the quasispecies population during the WN virus transmission cycle.

This study and the previous analyses of WN virus diversity in the US have primarily examined strains isolated after limited local transmission—isolates which represent the forward edge of WN virus movement in North America. Continued study of WN virus in now endemic areas of the eastern United States, where virus has circulated for three summers, and as it becomes established in areas of the southern and western United States that are endemic for St. Louis encephalitis virus, may reveal the emergence of more divergent variants as the virus contends with a partially immune host population.

Materials and methods

Dead bird and mosquito pool samples were obtained from the Harris County Mosquito Control Division, the Texas Department of Health, and the Galveston County Mosquito Control District. Viral RNA was extracted directly from dead bird brain tissue or mosquito pool homogenates or from infected Vero cell culture supernatants using the QiaAmp viral RNA extraction kit (Qiagen Inc., Valencia CA) according to the manufacturer's protocol. Products corresponding to all or part of the viral premembrane/membrane (prM/M) and envelope (E) protein genes were reverse transcribed using AMV reverse transcriptase (Roche, Indianapolis IN) and PCR amplified using *Taq* polymerase (Roche) with three primer pairs WN401 (AAAAGAAAAGAGGAGGAAAG) and WN1219A (GTTTGTCATTGTGAGCTTCT); WN1101 (GATGAATATGGAGGCGGTCA) and WN1816A (CCGACGTCAACTTGACAGTG); WN1751 (TGCATCAAGCTTTGGCTGGA) and WN2504A (TCTTGCCGGCTGATGTCTAT). In addition, a region corresponding to the end of the NS5 gene and part of the 3′ noncoding region was amplified for some strains. PCR products of the appropriate size were gel purified using the QIAquick kit (Qiagen Inc.) and, when sufficient quantities of template were obtained, directly sequenced using the amplifying primers. When yields were insufficient for direct sequenc-

ing the PCR products were cloned into the pGEM-T Easy vector (Promega Corp.). In these cases, multiple clones were sequenced for each to obtain a consensus sequence. Sequencing reactions were performed using ABI PRISM Big Dye Terminator v3.0 cycle sequencing kits (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Mouse virulence studies with strains 114 and 476 were carried out in 3- to 4-week-old female NIH Swiss mice (Harlan, Indianapolis, IN). Groups of eight mice were inoculated ip with serial 10-fold dilutions (0.1 to 10,000 PFU) of WN virus strain 382–99, 114, or 476.

Acknowledgments

The authors thank Darci Smith for assistance with some studies; Steve Smith, Esther Surriga, and Steve Burkett from the UTMB Biomolecular Resource Facility DNA sequencing laboratory for sequencing support; and Tom Solomon and Mike Holbrook for critical reading of the manuscript. This study was supported in part by NIH Grant AI-10984, CDC Cooperative Agreements U90/CCU620916-01 and U50/CCU620539-01, and funding from the State of Texas Advanced Research Program.

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